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The mechanism of inhibition of cancer cell proliferation by vitamin A is poorly understood because many of the targets that mediate the retinoid-dependent growth suppression are not known. We have recently identified a novel retinoid-responsive gene target, **TIG3**, that we believe may be a key player in mediating the retinoid-dependent suppression of tumor cell proliferation. Understanding the mechanism of TIG3 action may provide insights that lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation – the major goal of this proposal. ***Specific Aim 1*** Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to localize TIG3 in breast cancer cells. ***Specific Aim 2*** Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. We are testing this hypothesis. ***Specific Aim 3*** The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. A major goal of the study is to identify these targets. During the first year we have 1) constructed a plasmid-based TIG3 expression systems and used it to express TIG3 in cells, 2) identified a perinuclear localization of TIG3 in cells, 3) demonstrated that the TIG3 carboxy-terminal hydrophobic domain guides appropriate subcellular localization, 4) shown that the TIG3 carboxy-terminal tail is required for optimal cell killing, and 5) constructed an adenovirus expression system that permits efficient TIG3 expression for biochemical studies.

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INTRODUCTION

Breast cancer is a serious disease that effects millions of women. Vitamin A and related retinoids are important dietary components that are important for normal epithelial cell proliferation and differentiation and are thought to be chemopreventive against development of cancer. Analogs of vitamin A have been shown to be efficacious for treatment in the mammary gland and breast cancer systems, and have been shown to inhibit the proliferation of cultured breast cancer cells. However, the mechanism of inhibition of cancer cell proliferation by retinoids is poorly understood because many of the targets that mediate the retinoid-dependent growth suppression are not known. We have recently identified a novel retinoid-responsive gene target, **TIG3**, that we believe may be a key player in mediating the retinoid-dependent suppression of tumor cell proliferation. Treatment of $T_{47}D$ human breast cancer cells with retinoid increases TIG3 mRNA levels, an increase that is associated with a suppression of cell proliferation. TIG3 mRNA level, in contrast, is not increased nor is growth suppressed in a retinoid-insensitive line derived from MCF-7 cells. These results suggest that TIG3 may mediate the retinoid-dependent suppression. For these reasons we hypothesis that TIG3 may be a key mediator of retinoid-dependent tumor suppression in breast cancer. Understanding the mechanism of TIG3 action may provide insights that lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation – the major goal of this proposal. ***Specific Aim 1*** Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to use immunological and cell fractionation methods to localize TIG3 in breast cancer cells. ***Specific Aim 2*** Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. To test this, we will construct a series of mutants and measure the ability of each mutant to suppress growth using a breast cancer cell colony formation assay. ***Specific Aim 3*** The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation, affinity chromatography and two-hybrid screening. The candidate target proteins will be identified by peptide microsequencing (affinity chromatography) or gene cloning and sequencing (two-hybrid cloning). It is expected that these studies will reveal important clues about the TIG3 mechanism of action. It is our hope that understanding this how TIG3 signals growth suppression will lead to innovative new anti-breast cancer therapies.

BODY

Specific Aims 1 & 2

- *Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to use immunological and cell fractionation methods to localize TIG3 in cells.*
- *Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. To test this, we will construct a series of mutants and measure the ability of each mutant to suppress growth using a breast cancer cell colony formation assay.*

The studies outlined in the first two specific aims are most efficiently described when combined. Thus, we will describe the construction of our initial set of mutants, their detection in cells, and their subcellular distribution in this section.

Construction of TIG3 mutants TIG3 is an 18 kDa 164 amino acid growth suppressor protein that is present in very low levels in cells. An important goal of this study is to identify the role of various functional domains within the protein and to determine which domain controls subcellular localization. The sequence of TIG3 is shown in **Fig. 1**. The protein is divided into an amino-terminal domain and a carboxy-terminal hydrophobic domain. We hypothesized that TIG3 subcellular distribution is controlled by the carboxy-terminal hydrophobic domain and that this serves to anchor TIG3 to membranes. To test this idea we constructed the mutants shown in **Fig. 2**. $TIG3_{1\text{-}164}$ encodes the full length protein, while $TIG3_{1\text{-}134}$ encodes the TIG3 amino-terminus but lacks the carboxy-terminal tail.

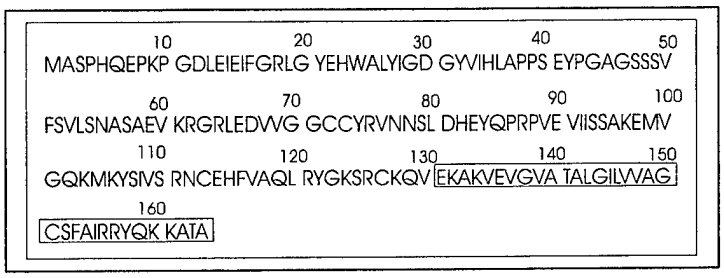

Fig. 1 Structure of TIG3. The amino terminal segment encodes amino acids 1-134, while the carboxy terminal tail includes amino acids 135-164.

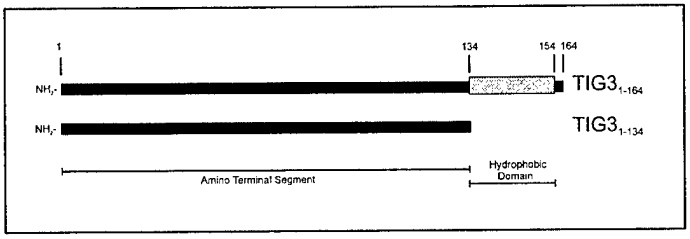

Fig. 2 TIG3 expression plasmids. The various TIG3 encoding segments were synthesized using PCR and then cloned into the CMV-powered expression plasmid, pcDNA3. The resulting plasmids, pcDNA3-$TIG3_{1\text{-}164}$ and pcDNA3-$TIG3_{1\text{-}134}$ encode, respectively, TIG3 (full length) and TIG3 lacking the carboxyl-terminal tail.

TIG3 subcellular localization- immunofluorescent detection For microscopy studies, cells were grown on Permano chamber slides (Nalge Nunc), transfected with the appropriate plasmid, encoding $TIG3_{164}$ (full length) or $TIG3_{1\text{-}134}$ (carboxy terminal truncation) and incubated in media. The cells were maintained in the transfection mixture for 24 - 48 h before analysis, depending on cell type. In the cyclohexamide studies, at 24 hours post-transfection, cyclohexamide was added at a concentration of 200 mM for 2 h (17). The cells were then fixed in iced methanol for 20 min, washed with PBS, and blocked for 30 min in PBS supplemented with 10% goat serum. After an additional wash, the cells were incubated with anti-$TIG3_{1\text{-}134}$ or pre-immune serum (1:500) for 1 hr at 4 C. To detect primary antibody binding, the slides are incubated in the dark for 1 h with Oregon Green 514-linked goat anti-rabbit IgG secondary antibody (Molecular Probes), and washed three times with PBS and once with water. One drop of N-propyl galate was placed on the center of each slide and a coverslip was sealed in place with nail polish. For single cell immunofluorescence studies, slides were viewed at sixty-fold magnification using a water immersion lens at 488 nm excitation/510 nm emission wavelengths. For confocal microscopy, slides were viewed using a 100x oil immersion lens on a LSM410 Inverted Microscope using argon-krypton laser excitation at 488 nm and fluorescence above

500 nm was captured. A 512 x 512 resolution size was used, ith exposures collected at 0.7 sec scan times. The presented images are representative of at least three separate experiments in which at least ten fields were imaged.

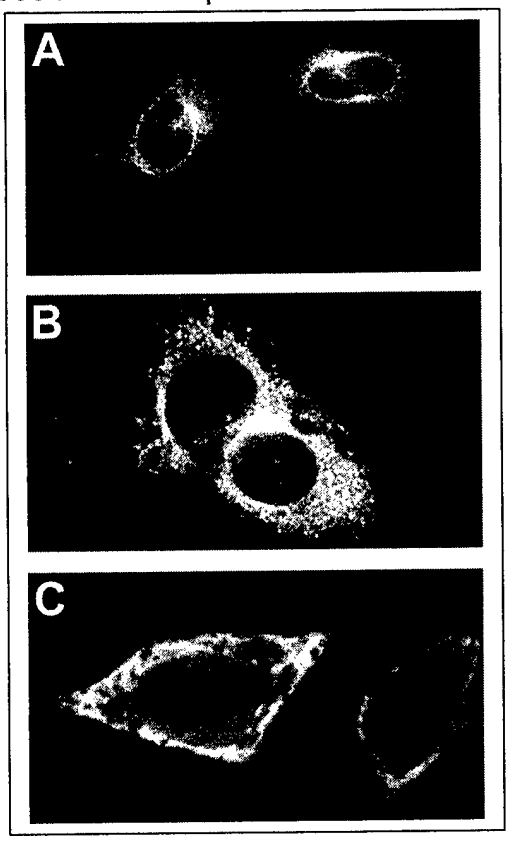

Fig. 3 Subcellular localization of $TIG3_{1-164}$ and $TIG3_{1-134}$ in CHO cells. Cells were transfected with expression plasmid encoding $TIG3_{1-164}$ (panels A and B) or $TIG3_{1-134}$ (panel B). Cells in panel B were additionally treated with cycloheximide as outlined in the text. CHO cells were chosen as an initial test cell since they are easily transfected, but similar distribution profiles are also observed for $T_{47}D$ cells.

As shown in **Fig. 3**, transfection of cells with $TIG3_{1-164}$ results in detection of TIG3 immunoreactivity in the perinuclear region. Addition of cycloheximide (panel B), which inhibits new protein synthesis and permits existing protein to exit the Golgi apparatus, does not result in an alteration of distribution. Panel C shows the localization of $TIG3_{1-134}$, the mutant that lacks the c-terminal hydrophobic domain. It is clear that $TIG3_{1-134}$ has a different subcellular distribution. Instead of being distributed in a perinuclear location, it has a more uniform distribution in the cytoplasm. These results suggest that TIG3 is localized in perinuclear structures and that the location is determined, at least in part, by the carboxyl-terminal domain.

Function of $TIG3_{1-164}$ and $TIG3_{1-134}$ We next evaluated the role of these mutants in regulating cell proliferation. For these studies, we performed colony formation assays. In this assay, equal numbers of cells, plated in 35 mm dishes, are transfected with 2 μg of plasmid with 3 ml Fugene 6 (Boehringer Mannheim). After 24 h, the cells were trypsinized, split, and replated in selection medium containing 800, 900, or 1000 μg/ml of G418, respectively, for CHO, $T_{47}D$ and HaCaT cells. Surviving colonies were allowed to expand for two weeks until colonies of about 50 cells had formed. The cells were fixed, stained with hematoxylin, and counted. As shown in **Fig. 4**, $TIG3_{1-164}$ reduces the proliferation of several cell types, including CHO (a hamster fibroblast cell line), $T_{47}D$ (breast cancer cells), and HaCAT (skin cancer) cells. $TIG3_{1-134}$, although somewhat less effective as an inhibitor of growth than $TIG3_{1-164}$, was still able to inhibit proliferation. Thus, these results argue that TIG3 is able to inhibit proliferation of breast cancer cells and other cells types. Moreover, the intact protein is an efficient inhibitor, but the amino terminal segment of the protein can also inhibit cell growth.

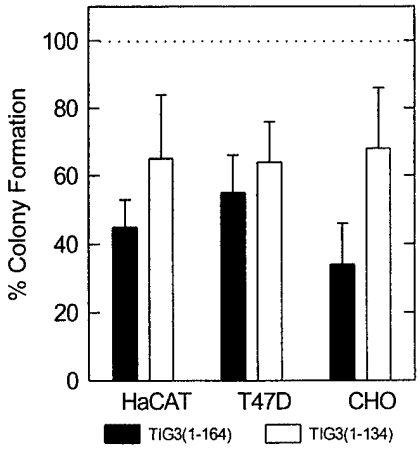

Fig. 4 Regulation of cell number by TIG3 proteins. Cells were transfected with $TIG3_{1-164}$ or $TIG3_{1-134}$ and then colony formation was monitored as outlined in the test.

Localization of green fluorescent protein (GFP)-TIG3 fusion proteins As an alternate method of monitoring distribution, we constructed expression vectors encoding GFP-$TIG3_{1-164}$ and GFP-$TIG3_{1-134}$ fusion proteins. pEGFP-C3 (Clonetech) was used as the parent vector for construction of plasmids designed to express GFP-TIG3 fusion proteins in eukaryotic cells. Green fluorescent protein (GFP) fusion protein-encoding plasmids, pGFP-$TIG3_{1-164}$ and pGFP-$TIG3_{1-134}$ were generated. A common upstream primer containing a *Hind*III site adjacent the translation start codon (5'-CAAGCTTATGGCTTCGCCAC ACCAAGAGCC) was used to generate both inserts. This primer was paired with a downstream primer (5'-GAGGATCCTCAGGCTGTTGCTTTTTTTTTTGGTATC), containing a *Bam*HI site adjacent the termination codon, to generate $TIG3_{1-164}$. For $TIG3_{1-134}$, the downstream primer was 5'-CGCTCGAGTCAC TTGGCCTTTTCCACCTG) encoding an *Xho*I site preceded by a translation termination codon. The inserts were placed into pZERO at the *Eco*RV site and sequenced. The $TIG3_{1-164}$ and $TIG3_{1-134}$ inserts were excised, respectively, with *Hind*III/*Bam*HI and HindIII/XhoI and transferred to pEGFP digested with the corresponding enzymes, to yield pGFP-$TIG3_{1-164}$ and pGFP-$TIG3_{1-134}$. In these plasmids, TIG3 is fused to the carboxyl terminus of green fluorescent protein.

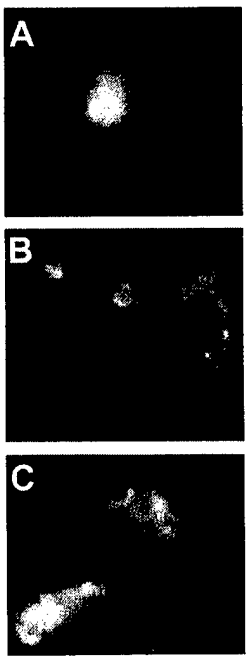

Fig. 5 Subcellular location of GFP-TIG3 fusion proteins. CHO cells were transfected with plasmids encoding GFP (panel A), GFP-$TIG3_{1-164}$ (panel B), and GFP-$TIG3_{1-134}$ (panel C). Equal numbers of cells, plated in 35 mm^2 dishes, were transfected with 2 µg of the appropriate GFP-TIG3 fusion encoding plasmid using the Fugene 6 transfection protocol (Boehringer Mannheim). Fluorescence was monitored at 48 h post-transfection on living cells.

These plasmids were then used to transfect cells to produce GFP-TIG3$_{1-164}$ and TIG3$_{1-134}$ fusion proteins in cells. As shown in **Fig. 5**, GFP is detected in a cytoplasmic distribution (panel A). In contrast, GFP-TIG3$_{1-164}$ is detected in a perinuclear location. GFP-TIG3$_{1-134}$ is detected in a cytoplasmic localization that is not distinguishable from the distribution of GFP.

These studies indicate that the TIG3 carboxy-terminal segment (amino acids 135-164) is responsible for the localization to the perinuclear location and that even when the TIG3 protein is fused to a larger soluble proteins (GFP), the carboxyl terminal segment still controls the localization. In contrast, the carboxyl terminal truncation mutant (TIG3$_{1-134}$) does not maintain this localization.

Specific Aim 3

- *The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation, affinity chromatography and two-hybrid screening.*

Development of an adenovirus-based TIG3 expression system We have hypothesized that TIG3 binds to target proteins to regulate cell proliferation and survivable. A major goal of this study is to identify these targets that TIG3 binds to as part of its role in regulating cell function. We initiated these studies by expressing TIG3 in cells and preparing cell extracts that could be used for TIG3 antibody pull down experiments. However, a major problem with these studies was the inability to express enough TIG3 protein to make these biochemical experiments possible. In addition, TIG3 kills cells very efficiently and limits the number of TIG3-expressing cells that can be obtained (i.e., they die to fast). To circumvent these difficulties we switched from the plasmid-based to an adenovirus-based expression system. The use of this system has solved the problem of minimal protein expression and the difficulty with maintaining cell viability.

In this system, TIG3 isoforms are expressed from an inducible adenovirus expression virus. This virus does not produce TIG3 unless a helper virus that encodes a transcriptional activator is present. Thus, we infect cells at 100% efficiency with adeno-TIG3, but there is no TIG3 produced until the helper virus is added. Upon addition of the helper virus, the cells produce larger amounts of TIG3 protein. We are currently examining the ability of this system to kill breast cancer cells. However, as shown in **Fig. 6**, we have determined that the virus does produce detectable TIG3 in MCF7 cells.

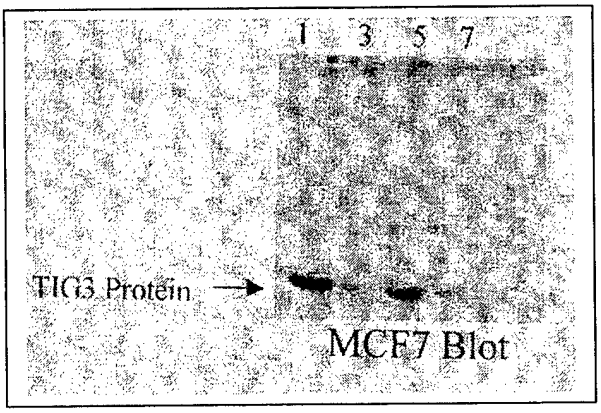

Fig. 7 Detection of TIG3 in adeno-TIG3 infected MCF7 cells. The cells were infected with TIG3-encoding adenovirus and after 48 h the cells were lysed and TIG3 levels were assayed by immunoblot. Lanes 1, 3, 5 and 7 indicate the level of expression in four separate infection experiments. The amount of virus added to the cells was reduced in experiments 3 and 7. Thus, TIG3 expression correlates with the amount of virus added.

KEY RESEARCH ACCOMPLISHMENTS

- We have constructed plasmid-based TIG3 expression systems and used these to express TIG3 in cells (*Specific Aims 1 and 2*)
- We have constructed our first TIG3 mutant, $TIG3_{1-134}$ and begun to study its function (*Specific Aims 1 and 2*)
- We have shown that TIG3 assumes a perinuclear location in cells (*Specific Aim 1*)
- We have demonstrated that the TIG3 carboxy-terminal hydrophobic domain is required for appropriate subcellular localization (*Specifc Aim 1*)
- Eliminating the TIG3 carboxy-terminal tail reduces the ability of TIG3 to kill cells (*Specific Aim 2*)
- TIG3 kills breast cancer cell lines (e.g., $T_{47}D$) (*Specific Aim 2*)
- Adenovirus expression systems have been constructed that permit more efficient studies of cell killing and permits efficient production of TIG3 in cells for biochemical studies (*Specific Aim 3*). This virus produces high level expression of TIG3 in MCF7 cells.

REPORTABLE OUTCOMES

- We have a manuscript in preparation that will be submitted later this year
- Anne Deucher and Shervin Dashti will be reporting on these TIG3 studies as part of their Ph.D. theses
- We have developed adenoviral TIG3-producing vectors that may be useful for gene therapy

CONCLUSIONS

We consider this work to be very important from the point-of-view of future breast cancer therapeutics. Our studies completed to date clearly show that TIG3 inhibits the breast cancer cell proliferation. We also suspect that TIG3 has the ability to kill (cause apoptosis) breast cancer cells independent of its effects on cell proliferation. We expect that by the time these studies are concluded we will have isolated the active domains within the protein that are responsible for these events, and that we will have also tested the ability of TIG3 to kill cells *in vivo* in a human tumor cell model system.

REFERENCES

Because this work is new and TIG3 has not been extensively studied in the past, there are no specific referenced manuscripts that need be cited.